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Potential of 2,4-Dihydroxybenzoic Acid as an Oviposition Stimulant for Mass-Reared Ladybird Beetles

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Abstract

The discovery of inexpensive, readily available bioflavonoids, and their degradation products that boost the reproductive potential of mass-reared predators is the overarching goal of this research. We tested the hypothesis that 2,4-dihydroxybenzoic acid (DHBA), an inexpensive degradation product of morin (a flavonol bioflavonoid), stimulates oviposition by the ladybird beetle *Coleomegilla maculata* (DeGeer). We also tested the hypothesis that *C. maculata* females must touch or taste DHBA to stimulate oviposition. We setup bioassays in communal cages (housing 10 females) and solitary cages (housing 1 female). In communal cages, nearly all egg clutches were found in or near the chemical dish with DHBA only. Provisioning cages with a tissue substrate reduced oviposition in the chemical dish. Regardless of oviposition site, egg number per clutch did not increase in communal cages or solitary cages with DHBA only. Affixing DHBA to the base of the chemical dish, then covering it with a nylon screen, reduced oviposition. This study suggests that females must touch or taste DHBA to stimulate oviposition. The physiological mechanism involved in oviposition stimulation requires further study. DHBA could potentially serve as a weak oviposition stimulant for predatory ladybird beetles in some mass-rearing systems.

Key words: biocontrol, flavonoid, morin, quercetin, rearing

A major challenge to mass rearing predators in high quantities is stimulating females to oviposit their full potential of eggs when reared on alternative foods (Riddick 2009; Sun et al. 2017; Riddick et al. 2018a,b). Also, chemical cues that females use to choose oviposition sites are often associated to natural prey, e.g., aphids and/or host plants under natural conditions in the field (Honěk 1980, Hodek and Evans 2012). In mass rearing systems, devoid of natural prey and host plants, many of these cues are lacking. In the absence of oviposition cues, females might lay fewer egg clutches and resort to laying their eggs on other substrates, such as tissue paper, filter paper, and aluminum sheets (Takahashi 1986, Allen and Riddick 2012, Hesler et al. 2012, Morales-Ramos and Rojas 2017, Riddick et al. 2018b). In the absence of any oviposition substrates, females lay their eggs on the underside of the lid or sidewalls of rearing containers (Iperti and Quilici 1986, Sakuratani and Nakamura 1997, Riddick et al. 2018b).

The identification and use of oviposition cues from nonprey and nonhost plants could improve predator reproduction in rearing systems devoid of oviposition substrates. A limited number of plant-derived chemical cues stimulate oviposition in predators, including ladybirds. For example, chemical cues from European barberry (*Berberis vulgaris* L., Ranunculales: Berberidaceae) crude extracts stimulated oviposition in two ladybird beetles, *Adalia bipunctata* (L.)

and *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) (Shah 1983). Chemical cues from eastern redcedar (*Juniperus virginiana* L. [Pinales: Cupressaceae]) crude extracts and fractions stimulated oviposition in *A. bipunctata* and three other ladybird species, *Coccinella transversoguttata* Faldermann, *Cycloneda munda* (Say), and *Coleomegilla maculata* (DeGeer) (Coleoptera: Coccinellidae) (Boldyrev et al. 1969, Smith et al. 1973). Polyphenols in redcedar wood were the compounds responsible for oviposition stimulation (Boldyrev et al. 1969, Smith et al. 1973). Several fractions isolated from redcedar heartwood stimulated oviposition in *C. maculata* in cage bioassays; some of the active fractions were bioflavonoids, such as taxifolin, quercetin, and naringenin, to a lesser extent (Riddick et al. 2018a).

In the context of this research, we propose that an effective chemical stimulant should cause females to 1) oviposit in close proximity to the chemical source and 2) produce more egg clutches. As a continuation of our search for effective oviposition stimulants, derived from plant sources, for mass-reared ladybird beetles, we considered the possibility of using bioflavonoid degradation products. When exposed to atmospheric oxygen, bioflavonoids could possibly degrade to smaller molecules, such as aromatic carboxylic acids containing two hydroxyl groups (Makris and Rossiter 2001, 2002). One or more of these lower molecular weight compounds could be

alternative, less expensive, oviposition stimulants. Based on availability (at Sigma–Aldrich), cost, and safety, we selected an aromatic carboxylic acid, 2,4-dihydroxybenzoic acid (DHBA) for testing. DHBA has a molecular weight of 154.12 g/mol. It is a degradation product of morin, a flavonol bioflavonoid (Makris and Rossiter 2002), which has a molecular weight of 302.24 g/mol. According to the Sigma–Aldrich online catalog (https://www.sigmaaldrich.com), 5 g of morin (hydrated form) is $43.50, but 100 g of DHBA is $25.40 USD. In comparison, 100 g of quercetin is $207.00 USD. Thus, DHBA is 8.1-fold less expensive than quercetin at the 100-g quantity.

Quercetin is also a flavonol bioflavonoid; it is chemically similar (same chemical formula, molecular weight, and appearance) to morin. The two flavonols differ in the position of the two hydroxyl groups on one of the aromatic hydrocarbons. These two hydroxyl groups are in the 'ortho' and 'meta' positions in quercetin and morin, respectively.

DHBA is present in some fruits, medicinal plants, avocado, red wine, and other plants and plant products (Sanz et al. 2012, Ahmab et al. 2016, Feliciano et al. 2017). It has anticancer, antiparasite, and antibacterial properties (Alves et al. 2013, Zhao et al. 2014, Fueyo-Gonzalez et al. 2017). We did not find any reference to biological activity of 2,4-DHBA against insects or related arthropods in the literature. Information on the functionality of DHBA as an oviposition stimulant in any organism is unreported previously.

In this study, we tested the hypothesis that DHBA stimulates oviposition by *C. maculata*. We also tested the hypothesis that females must touch or taste DHBA to stimulate oviposition. The primary objectives were to determine the daily number of egg clutches (at different sites) and the number of eggs per clutch (combined sites) in solitary cages, housing only one female, and in communal cages, housing 10 females. In addition, we took a daily snapshot of the location of females in communal cages, to get some indication of the amount of time that females spend near the chemical (DHBA).

Materials and Methods

Insect Colonies and Food Sources

Coleomegilla maculata immatures and adults in our stock colony were reared separately in plastic containers in an environmentally controlled room (24°C, 50–60% RH, and 16-h photophase, year-round). Adults and larvae were fed an excess of frozen-fresh eggs of the Mediterranean flour moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), twice a week. A microcentrifuge tube containing distilled water (stoppered with a cotton wad) was present in rearing cages at all times. Please see Riddick et al. (2018b) for a more detailed description of the insect colony.

Description of DHBA and Source

DHBA, also known as β-resorcylic acid, has the chemical formula $C_7H_6O_4$ and molecular weight 154.12 g/mol. We purchased DHBA (97% pure powder, product no. D109401) from Sigma–Aldrich (St. Louis, MO).

DHBA on Oviposition Behavior and Female Location in Communal Cages

In a previous study, we gave a detailed description of the methodology used to test the oviposition responses and location of *C. maculata* females in 1-liter cages, with oviposition chambers inside (Riddick et al. 2018b). We tested the ovipositional responses of 10 females to DHBA in 1-liter 'communal' cages (see Fig. 1a–c). In

summary, two replicate 1-liter cages were used for all treatments, for a total of eight cages and 80 females. Ten females of approximately the same age (i.e., 40-d old) were randomly assigned to each treatment cage. Treatments included DHBA only, DHBA plus tissue (inside the chamber), DHBA plus tissue (outside the chamber), and tissue (inside the chamber) only. Each oviposition chamber held a stack of four Petri dishes, each containing a tiny chemical dish. The four chemical dishes contained DHBA powder (2 mg each) in the appropriate cage; the chemical dishes were empty in cages not containing any of this compound. We recorded the oviposition site preferences of females twice a day, once in the morning and again in the afternoon, by observing and counting the number of clutches at sites inside the cages. The sites included the chemical dish, cage wall, and food dish. We also noted the presence of egg clutches on tissue (inside and outside the chamber) in the respective treatment cages. We counted the number of eggs per clutch, regardless of oviposition site.

The location of females in each cage was recorded twice a day as well, once in the morning (at ~0900 h) and in the afternoon (at ~1600 h), as in a previous study (Riddick et al. 2018b). For ease of recording, and to limit the disturbance to females while checking the locations of the 10 females in each treatment cage, we considered only two distinct locations, 1) inside the oviposition chamber and 2) outside the oviposition chamber. Recording female location could provide clues to whether females remain inside or move outside the oviposition chamber (with DHBA) after oviposition ceases.

Cages were maintained in a growth chamber (24°C, 60% RH, 16-h photophase). Adult females were fed *E. kuehniella* eggs every other day; old food and feces were discarded every other day.

DHBA With or Without Nylon Cover on Oviposition Behavior in Solitary Cages

In our previous studies (Riddick et al. 2018a,b), females often physically contacted chemical powder (in the chemical dish, 1-cm high, 3.5-cm diam) during the course of the 12-d trials. As a consequence, we had to replenish the chemical dishes to ensure that approximately the same quantity of compound was always in the chemical dish. Also, females occasionally were seen tasting or ingesting quercetin and powdered fractions isolated from extracts of redcedar heartwood (Riddick et al. 2018a,b). To experimentally test whether or not females needed to touch or taste (gustatory response) rather than simply smell (olfactory response) DHBA to elicit a change in oviposition behavior, we placed a 1.2- × 1.2-cm (length by width) piece of double-sided, clear Scotch Magic tape in the center of the chemical dish, then carefully affixed 1 mg of DHBA powder onto the tape. Then, we gently pressed a similarly sized nylon screen (U.S. mesh size 18) on top of the powder. The setup of the test cages with DHBA covered with the nylon screen is illustrated in Fig. 2a and b. In the control cages, we also used the tape, but no chemical (i.e., no DHBA); then affixed the screen on top of the tape (Fig. 2b, image on the right). To recap, the treatments in this experiment included DHBA only, DHBA plus screen cover, and the screen cover only (control). We used seven replicate 250-ml 'solitary' cages (6 × 8-cm, height, diam) for all treatments. All cages contained a tiny Petri dish (a chemical dish, 1.0 × 3.5 cm) containing DHBA powder (1 mg) in the appropriate cage, with or without the nylon cover. The chemical dish was empty, but with the screen cover, in control cages. Females of the same approximate age (i.e., 40-d old) were randomly assigned to treatment cages, one female per cage.

We recorded the oviposition site preferences of females (as the number of clutches at the various sites in each cage), twice a day, for
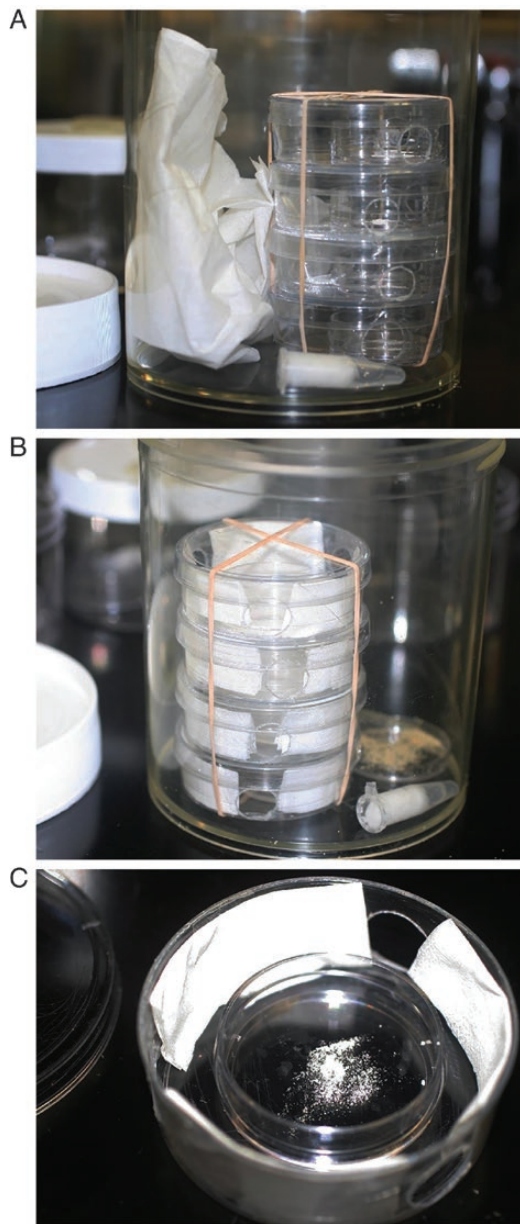
Fig. 1. (a) Lateral view of 1L communal cage with water tube, four-unit oviposition chamber, and tissue substrate outside chamber, (b) oviposition chamber with tissue substrate inside, and (c) close-up of individual unit, displaying tissue substrate, and DHBA powder in the chemical dish.

12 consecutive days. The sites included the chemical dish, cage wall, and food dish. We counted the number of eggs per clutch, regardless of oviposition site.

Cages were maintained in a growth chamber (24°C, 60% RH, 16-h photophase). Adult females were fed *E. kuehniella* eggs every other day; old food and feces were discarded every other day.

Statistical Analysis

The experimental design was a split plot with treatments arranged in a completely randomized design with replicate cages as sampling units in both the communal cage and solitary cage bioassays. In communal cages, the subunit was clutch site or female location; in solitary cages, the subunit was clutch site. Preliminary analysis indicated no effect of cage within each treatment. Therefore, the analysis of
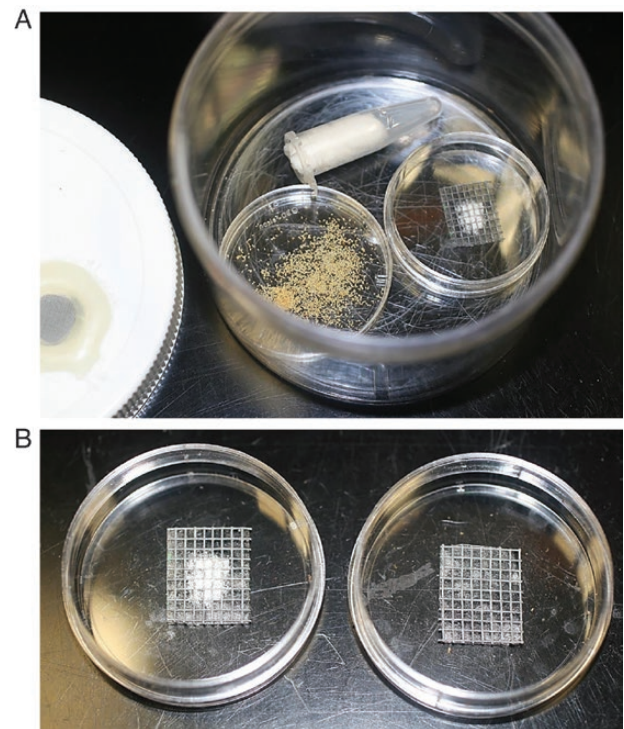

Fig. 2. (a) Dorsal view of 250-ml solitary cage with water tube, food dish, and chemical dish (with DHBA powder affixed to base of dish, then covered with nylon screen), and (b) close-up view of chemical dish with DHBA and screen cover (on left) or screen cover (affixed to the base of the dish) only (on right).

variance (two-way ANOVA) included fixed effects; treatment, clutch site (or female location), and their interactions. Note that tissue substrate was not included in the statistical analysis, because it was not in the DHBA treatment cages, in the communal cage bioassays. (A tissue substrate was never used in the solitary cage bioassays.) The two-way ANOVA tested for significance of treatment on clutch number per site, in communal and solitary cages, and female location in communal cages. A one-way ANOVA tested for significance of treatment on egg number per clutch, regardless of clutch site. Treatment means were significantly different following the ANOVA, if $P < 0.05$. The LSD method (i.e., an extended Student's *t*-test) was used to separate means, when necessary. JMP 12.0.1 (2012, SAS Institute Inc., in Cary, NC) software assisted with the analysis of data.

Results

DHBA on Oviposition Behavior in Communal Cages

In communal cages, females preferred to oviposit in the chemical dish in cages with DHBA (in the oviposition chamber) than in cages without it (Fig. 3, Table 1). Approximately five clutches were laid each day by a combined 10 females in cages with DHBA only. In cages with DHBA plus tissue, inside the chamber, clutch number declined to an average of 4 in the chemical dish. Females rarely oviposited on the cage wall and never in the food dish in cages with DHBA or DHBA plus tissue, inside the chamber. Note that a few clutches were found on the tissue substrate, in the DHBA plus tissue (inside and outside) treatment cages. Clutch number was not significantly different between chemical dish and cage wall sites in DHBA plus tissue, outside cages (Fig. 3). Rarely did females oviposit in the chemical dish in the absence of DHBA, as observed in the tissue (inside) treatment cages. Instead, females oviposited on the tissue substrate.

Regardless of oviposition site, mean egg number per clutch did not differ significantly among treatments (Table 2). Mean egg number per clutch was 11.7 and 13.3 in cages with DHBA only and tissue only (inside), respectively. The CIs of the mean values indicated that egg number was not consistent within and among treatments.

DHBA on Female Location in Communal Cages

In the DHBA treatment cages, more females were observed outside (rather than inside) oviposition chambers; in the tissue (inside) treatment cages, more females were inside the oviposition chamber (Fig. 4). When comparing the location of females in cages among treatment groups, significantly more females were observed outside the oviposition chamber in the DHBA plus tissue (outside) treatment cages (Fig. 4, Table 1).
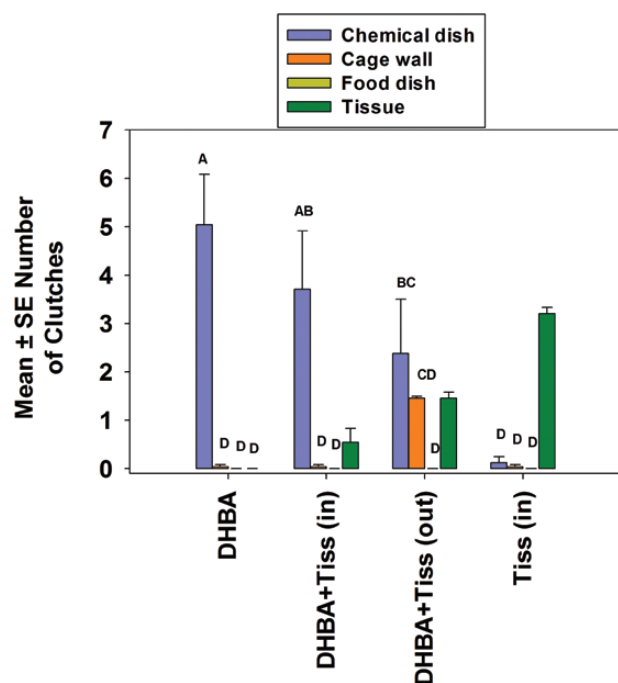

Fig. 3. Mean ± SE number of *C. maculata* egg clutches at oviposition (clutch) sites in communal cages (housing 10 females) as affected by DHBA and a tissue substrate in experiment 1. Letters above graph bars reflect mean differences in the interaction of treatment and site, only.

DHBA With or Without Cover on Oviposition Behavior in Solitary Cages

In solitary cages containing DHBA only, females preferred to oviposit in the chemical dish rather than the cage wall or food dish (Fig. 5, Table 1). Approximately 0.6 and 0.2 clutches per day per female were found in the chemical dish and the cage wall, respectively, in cages with DHBA only. When the nylon screen covered DHBA, females preferred to oviposit on the cage wall. In cages lacking DHBA (i.e., screen cover only), females preferred to oviposit on the cage wall (Fig. 5). Females rarely laid clutches in the food dish in any of the treatment cages.

Irrespective of oviposition site, egg number per clutch was not significantly different among the treatments (Table 2). The mean egg number per clutch in the DHBA-only and screen-only treatment cages was 12.1 and 11.0 eggs, respectively. CIs of the mean indicated that egg number was not consistent within and among treatments.

Discussion

This study highlights the potential of DHBA as a weak oviposition stimulant for *C. maculata* because it causes females to oviposit in specific locations but does not increase daily egg clutch production. To some extent, it compares favorably with quercetin as an oviposition stimulant, in its capacity to alter oviposition site selection. However, *C. maculata* females produced more egg clutches when quercetin and a tissue substrate were in close proximity in one of two experiments (Riddick et al. 2018b). In this study, greater egg clutch production did not occur when DHBA and a tissue substrate were in close proximity, suggesting that DHBA would be less effective in a mass rearing system when tissue substrates were utilized. Despite this discrepancy, it could be more cost-effective to use DHBA, even if it is less effective than quercetin, because DHBA is 8.1-fold less expensive than quercetin.

This study cannot suggest that smaller molecules, perhaps a degradation product of one or more bioflavonoids, are actually the true source of oviposition stimulation in ladybirds (e.g., *C. maculata*). Further testing with a range of other degradation products (of bioflavonoids) are necessary. Moreover, the other structural isomers of dihydroxybenzoic acid could be tested for their stimulatory capacity.

In regard to efficient mass rearing, grouping females into communal cages would be a space-saving technique. In the communal cage experiment, the majority of egg clutches were found in or near the chemical dish containing DHBA, when there was no tissue substrate anywhere in the cages, suggesting that DHBA was an oviposition
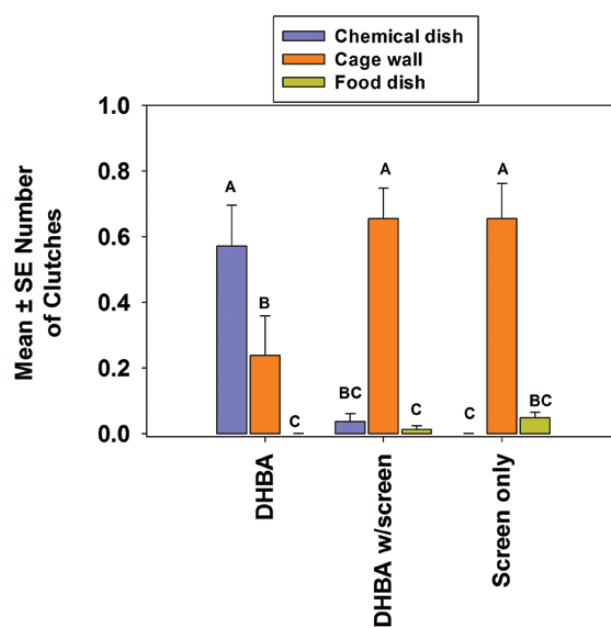
Table 1. ANOVA statistics of the interaction between treatment, clutch site, and female location in communal cages; treatment and clutch site in solitary cages

Experiment	Source of variation	F	df	P
1. Communal cage	Treatment and clutch site	5.32	6, 12	0.007
	Treatment	4.68	3, 12	0.02
	Clutch site	29.02	2, 12	<0.0001
1. Communal cage	Treatment and female locale	41.9	3, 8	<0.0001
	Treatment	0.0	3, 8	1.0
	Female locale	17.3	1, 8	0.003
2. Solitary cage	Treatment and clutch site	14.03	4, 54	<0.0001
	Treatment	0.22	2, 54	0.80
	Clutch site	33.18	2, 54	<0.0001

Ten females were inside two replicate 1-liter communal cages per treatment; one female was inside seven replicate 250-ml solitary cages per treatment. Refer to Figs. 3 and 5 for graphical displays of clutch site selection in experiment 1 and experiment 2, respectively. Data were pooled over consecutive days and averaged per treatment group. Sample sizes were 24 and 16 observations for the clutch site and female locale analyses, respectively, in communal cage design; 63 observations in solitary cage design. *P* < 0.05 indicates significant differences among sources of variation.

Table 2. Effects of treatments on mean ± SE number of *C. maculata* eggs per clutch per female per day, irrespective of oviposition (clutch) site

Experiment	Treatments	Number of eggs per clutch	CI (95%), Lower, Upper
1. Communal cage	DHBA	11.68 ± 0.91a	9.66, 13.70
	DHBA w/ tissue (in)	11.10 ± 0.28a	9.08, 13.12
	DHBA w/ tissue (out)	12.16 ± 1.06a	10.14, 14.18
	Tissue (in)	13.29 ± 0.29a	11.27, 15.31
	$F = 1.62$		
	$df = 3, 4$		
	$P = 0.32$		
2. Solitary cage	DHBA	12.08 ± 0.65a	9.97, 14.18
	DHBA w/ screen	13.14 ± 1.33a	11.04, 15.25
	Screen only	11.04 ± 0.90a	8.93, 13.14
	$F = 1.10$		
	$df = 2, 18$		
	$P = 0.35$		

Ten females were inside two replicate communal (1L) cages per treatment for 12 consecutive days; one female was inside seven replicate solitary (250 ml) cages per treatment for 12 d. Data were pooled over consecutive days and averaged per treatment group. Sample sizes were 8 observations in the communal cage experiment and 21 observations in the solitary cage experiment. An *F*-test (one-way ANOVA) was used for data analysis. The same alphabet after the mean value in a column indicates no significant difference ($P > 0.05$) between treatments. CI represents the lower and upper 95% CI of the mean.
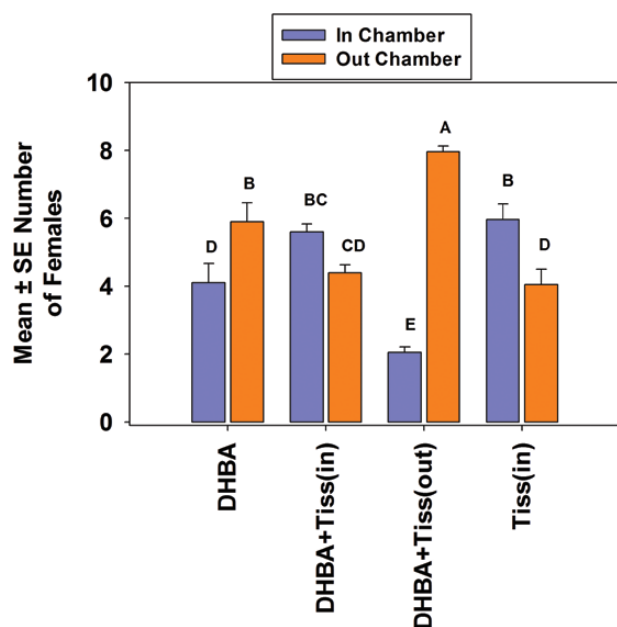
Fig. 4. Mean ± SE number of *C. maculata* females inside or outside oviposition chamber in communal cages (housing 10 females) in response to treatments in experiment 1. Letters above graph bars reflect mean differences in the interaction of treatment and location, only.

Fig. 5. Mean ± SE number of *C. maculata* egg clutches at oviposition (clutch) sites in solitary cages (housing 1 female) in relation to DHBA covered with nylon screen (or not) in experiment 2. Letters above graph bars reflect mean differences in the interaction of treatment and site, only.

stimulant, in part. The functionality of DHBA (or any of its structural isomers) as an oviposition stimulant for any organism has not been reported before, to our knowledge. The decline in the preference for the chemical dish (with DHBA) in the presence of tissue indicates that tissue paper is also stimulatory. Indeed, tissue paper is routinely utilized in our ladybird rearing operation and females used in this experiment had been exposed to tissue substrates for a few weeks before experimentation. Moreover, the surface area was greater on the tissue substrate than the small dishes (containing DHBA) in communal cages. If surface area is positively related to oviposition frequency, it is conceivable that more females would prefer to oviposit near DHBA, rather than on tissue, if the surface area occupied by DHBA was equivalent to that of the tissue substrate.

Our experiments also indicated that females tended to 'rest' on the tissue substrate in communal cages when DHBA was present or absent suggesting that chemical cues on the tissue were attractive to females or simply because the tissue provided a more secure grip for their tarsi than the smooth plastic surfaces of the cage. This behavior was observed previously as *C. maculata* females preferred to rest on tissue in communal cages with or without quercetin (Riddick et al. 2018b). These observations suggest that females will tend to oviposit near DHBA then move away from it after oviposition ceases. Moving away from the egg clutches would be advantageous in regard to preventing egg cannibalism by females.

In solitary cages, the majority of egg clutches were found in the chemical dish in cages with DHBA. This stimulatory effect

was reduced dramatically when females were unable to physically contact DHBA, suggesting that chemoreceptors, presumably on antennae, tarsi, and maxillary palpi, were involved in oviposition stimulation in *C. maculata* females.

Olfactory and tactile perception of plant-based chemical cues by ladybird beetles have been reported before (Hatano et al. 2008, Pettersson et al. 2008, Honěk 2012). Yet, the capacity of these plant cues to stimulate oviposition is less well known (Boldyrev et al. 1969, Smith et al. 1973, Shah 1983). More importantly, the functionality of olfactory chemoreceptors alone, or in combination with tactile and/or gustatory chemoreceptors, in oviposition stimulation has not been reported previously, to our knowledge.

Conclusion

This study provides evidence that DHBA has the potential to stimulate some *C. maculata* females to oviposit in cages in specific locations, i.e., Petri dish containing DHBA. In the presence of a tissue substrate, DHBA was less stimulatory. This study suggests that females must physically contact, and possibly ingest, DHBA before stimulation occurs. Future research should seek to reveal the physiological mechanism involved in oviposition stimulation in mass-reared ladybird beetles in the presence of DHBA and related compounds.

Acknowledgments

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